

Simultaneous silencing of β -catenin and signal transducer and activator of transcription 3 synergistically induces apoptosis and inhibits cell proliferation in HepG₂ liver cancer cells

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Abstract. The tumorigenesis and maintenance of a cancer cells is dependent upon the collaboration of multiple signaling pathways. Signal transducer and activator of transcription 3 (STAT3) and β -catenin are at the center of multiple cancer-associated signaling pathways; therefore, simultaneously targeting STAT3 and β -catenin may be a potential cancer treatment, leading to induced lethality of cancer cells. In the present study, HepG₂ liver cancer cells were transfected with small interfering RNA (siRNA) against β -catenin and STAT3 alone or in combination. The cell growth was assessed using an MTT assay and the levels of cell apoptosis were detected using flow cytometry. Protein levels of caspase-3, cleaved caspase-3, poly(ADP-ribose) polymerase (PARP) and cleaved PARP were determined using western blot analysis. Following siRNA transfection, β -catenin and STAT3 protein levels decreased at 72 h. HepG₂ cell growth inhibition and early apoptosis in the β -catenin and STAT3 siRNA co-transfection group were significantly greater than those in the groups transfected with β -catenin or STAT3 siRNA alone. Decreased caspase-3 and PARP levels, as well as enhanced cleavage of caspase-3 and PARP were observed in the β -catenin and STAT3 co-transfection group. Simultaneous silencing of β -catenin and STAT3 using siRNAs resulted in an enhanced loss of cell viability and induction of apoptosis in HepG₂ liver cancer cells, suggesting that these genes are promising targets for the further preclinical and clinical development of

anti-cancer therapeutic strategies, which target several cancer signaling pathways simultaneously.

Introduction

Liver cancer is one of the most common types of cancer in humans globally (1). It is often diagnosed at an advanced stage and is resistant to the majority of treatment options currently available. Therefore, it is important to further investigate the biology of liver cancer and identify novel therapeutic strategies for the management of liver cancer.

Cancer results from a number of genetic and epigenetic alterations (2), which together lead to the deregulation of gene expression, protein interaction networks and cell metabolism. It is now widely accepted that the initiation, progression and maintenance of a cancer cell relies on the collaboration of multiple signaling pathways, which function simultaneously in a non-linear manner. Therefore, targeting a single gene or pathway may not be sufficient to eradicate cancer cells. It was previously identified that silencing a single β -catenin gene inhibited liver cancer cell growth but was not able to eliminate the liver cancer completely (3). Simultaneous inhibition of two or more key cancer growth-dependent signaling pathways may lead to the induced death of cancer cells.

Deregulation of the Wnt/ β -catenin and signal transducer and activator of transcription 3 (STAT3) signaling pathways has been observed in the majority of cancer types and is closely associated with the genesis and development of liver cancer. The multifunctional protein β -catenin is the activation center of the Wnt signaling pathway (4-6). The Wnt/ β -catenin signaling pathway is able to affect the growth of liver cancer through regulating the cell cycle, apoptosis, angiogenesis, telomerase activity and other cell growth signaling pathways (3,7). For example, pharmacological inhibition of the Wnt/ β -catenin signaling pathway promotes cell apoptosis in neuroblastoma cell lines (8). The phosphatase and tensin homolog/phosphoinositide 3-kinase/Akt and Wnt/ β -catenin signaling pathways are involved in the regulation of human colon cancer cell proliferation (9). The oncogene STAT3 is at the convergence of numerous tumor-associated tyrosine kinase signaling pathways; thus, it is important in the regulation

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of cancer cell growth and apoptosis. In addition, STAT3 is upregulated in numerous types of cancer (10,11). For example, inactivation of STAT3 signaling induced apoptosis in HCT116 human colon cancer cells (12) and inhibited the proliferation and metastasis of hepatocellular carcinoma (13). In addition, inhibition of STAT3 activation was reported to suppress the tumorigenicity and growth of nasopharyngeal carcinoma cells (14).

Regarding the importance of β -catenin and STAT3 in tumorigenesis, their synergistic role during the initiation, progression and maintenance of cancer was investigated. The aim of the present study was to investigate the levels of cell growth and apoptosis following simultaneous silencing of β -catenin and STAT3 genes by siRNAs in HepG₂ liver cancer cells.

Materials and methods

Cells and reagents. HepG₂ liver cancer cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's minimum Eagle's medium (DMEM) with high glucose (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% (vol/vol) fetal calf serum (FCS; TBD Biotechnology Corporation, Tianjin, China) at 37°C in a humidified, 5% carbon dioxide atmosphere. Lipofectamine™ 2000 was purchased from Invitrogen Life Technologies, and an apoptosis kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Small interfering RNAs (siRNA) against β -catenin or STAT3 were synthesized by Shanghai Genepharm (Shanghai, China). The siRNA sequences directed against the genes were as follows: β -catenin sense, 5'-GGGUUCAGAUGAUUAAAUUTT-3' and anti-sense, 5'-AUUUAUAUCAUCUGAACCCAG-3'; STAT3 sense, 5'-CAUCUGCCUAGAUCGGCUAdTdT-3' and anti-sense, 5'-UAGCCGAUCUAGGCAGAUGdTdT-3'. The scrambled siRNA control sequence was: sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and anti-sense, 5'-ACGUGACACGUUCGGAGAATT-3'. The mouse monoclonal β -actin (1:200; cat. no. sc-47778), mouse monoclonal β -catenin (1:200; cat. no. sc-7963) and rabbit polyclonal STAT3 (1:200; sc-7179) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The rabbit polyclonal caspase-3 (1:1,000; cat. no. 9662), rabbit monoclonal cleaved caspase-3 (1:1,000; cat. no. 9664), rabbit polyclonal poly(ADP-ribose) polymerase (PARP; 1:1,000; cat. no. 9542) and rabbit monoclonal cleaved PARP (1:1,000; cat. no. 5625) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:5,000; cat. no. sc-2005) and goat anti-rabbit (1:5,000; sc-2004), were also purchased from Santa Cruz Biotechnology, Inc.

Transient transfection. A total of 3×10^5 cells were plated in six-well plates in triplicate and grown to 30-50% confluency. For transfection, 100 nM siRNA in 10 μ l Lipofectamine™ 2000 was administered to each well containing 2 ml DMEM without FCS. At 6 h after the transfection, the media was replaced with DMEM containing 10% FCS. After 72 h, the cells were harvested and the proteins were isolated using radioimmunoprecipitation assay buffer (Solarbio Technology, Beijing,

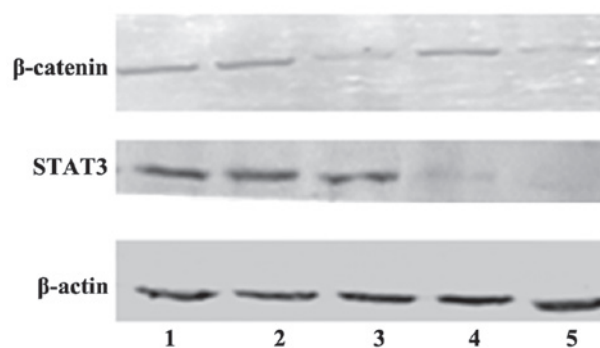


Figure 1. β -catenin and STAT3 silencing by siRNAs in HepG₂ cells. HepG₂ cells were transfected with β -catenin siRNA and STAT3 siRNA alone or in combination using Lipofectamine™ 2000 for 72 h. β -catenin and STAT3 proteins were determined by western blot analysis with β -actin as the loading control. 1, Control group; 2, negative siRNA control group; 3, β -catenin transfection group; 4, STAT3 transfection group; 5, β -catenin and STAT3 co-transfection group. STAT3, signal transducer and activator of transcription 3; siRNA, small interfering RNA.

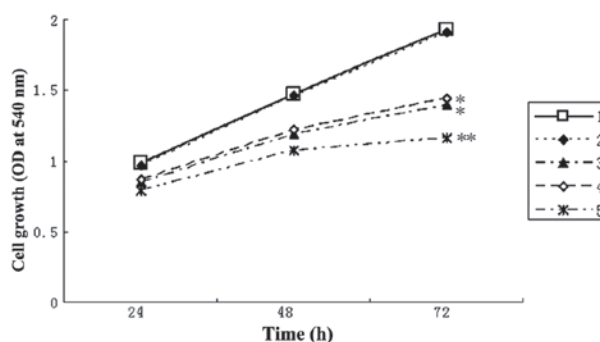


Figure 2. Simultaneous inhibition of β -catenin and STAT3 results in enhanced reduction of cell viability of HepG₂ cells. HepG₂ cells were transfected with β -catenin siRNA and STAT3 siRNA alone or in combination with Lipofectamine™ 2000 for the indicated times. The cell viability was assessed using an MTT assay. 1, Control group; 2, negative siRNA control group; 3, β -catenin transfection group; 4, STAT3 transfection group; 5, β -catenin and STAT3 co-transfection group. * $P < 0.05$, compared with the control group; ** $P < 0.05$, compared with the control group and the groups transfected with β -catenin or STAT3 alone. STAT3, signal transducer and activator of transcription 3; siRNA, small interfering RNA; OD, optical density.

China) supplemented with a protease inhibitor (phenylmethylsulfonyl fluoride; Solarbio Technology) and phosphatase inhibitors (Applygen Technologies, Inc., Beijing, China). In the co-transfection group, 100 nM siRNA against β -catenin and 100 nM siRNA against STAT3 were transfected into cells with Lipofectamine™ 2000 according to the manufacturer's instructions. All experiments were performed in triplicate and representative results are presented.

Determination of hepatocellular carcinoma (HCC) cell growth using an MTT assay. A total of 1.5×10^4 cells were plated in 96-well plates in triplicate and were grown to 30-50% confluency at the time of transfection. The siRNA-Lipofectamine™ 2000 complex (50 μ l) was administered to each well containing 100 μ l DMEM without FCS. The medium was replaced with DMEM containing 10% (vol/vol) FCS at 6 h of transfection. MTT (20 μ l; 5 mg/ml; Solarbio Technology) diluted in phosphate-buffered saline (PBS; Beyotime Institute

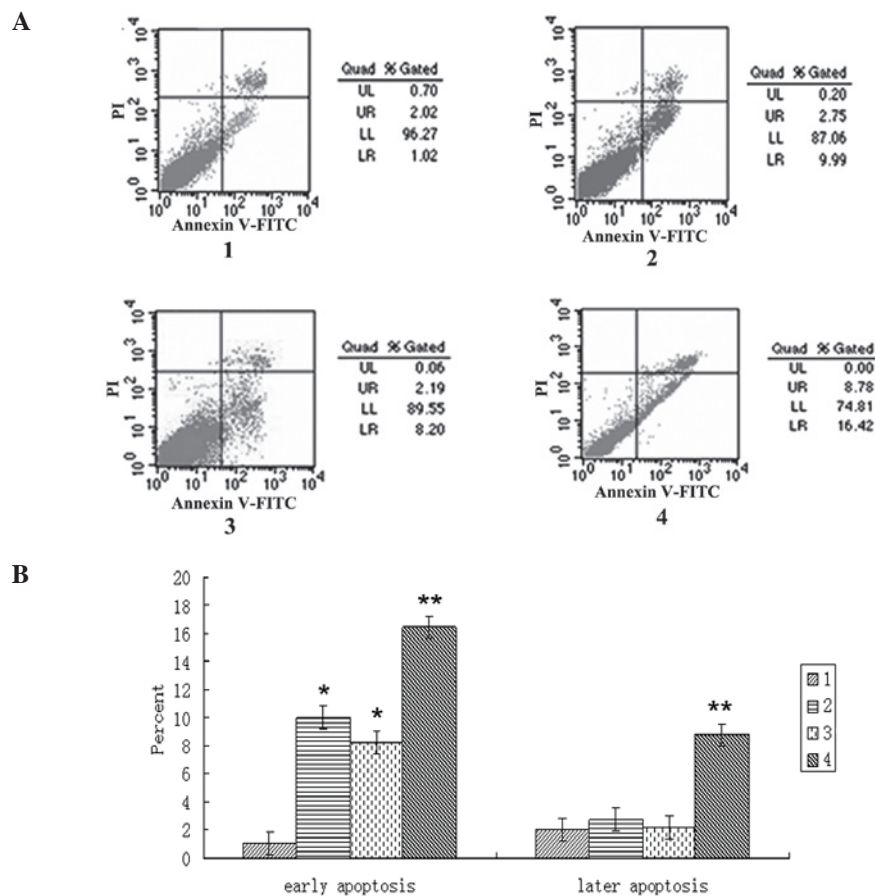


Figure 3. Co-transfection of siRNAs targeting β -catenin and STAT3 leads to increased apoptosis in HepG₂ cells. HepG₂ cells were transfected with β -catenin siRNA and STAT3 siRNA alone or in combination using Lipofectamine™ 2000 for 72 h. Cell apoptosis was determined by PI and annexin V-FITC staining followed by flow cytometric analysis. (A) Viable cells were negative for PI and annexin V-FITC, apoptotic cells were positive for annexin V-FITC and negative for PI, whereas late apoptotic/dead cells were positive for annexin V-FITC and PI staining. Non-viable cells, which underwent necrosis, were positive for PI but negative for annexin V-FITC. In the scatter diagram, the upper left quadrant represents mechanically damaged cells, the upper right quadrant represents late apoptotic cells, the lower left quadrant represents viable cells and the lower right quadrant represents early apoptotic cells. (B) Data were normalized to an internal control and expressed as the mean \pm standard deviation. The Y-axis indicates the ratio of early and late apoptosis, respectively. * $P < 0.05$, compared with the control group; ** $P < 0.05$, compared with the control group and the groups transfected with β -catenin or STAT3 alone. 1, Control group; 2, β -catenin transfection group; 3, STAT3 transfection group; 4, β -catenin and STAT3 co-transfection group. STAT3, signal transducer and activator of transcription 3; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; PI, propidium iodide.

of Biotechnology, Shanghai, China) was added to the medium at 24, 48 and 72 h after the transfection. After an incubation for 4 h, the medium was removed and the cells remained at the bottom of the wells. Dimethyl sulfoxide (200 μ l; Solarbio Technology) was added to each well to dissolve the formazan crystals in the cells. The absorbance was measured using a microplate reader (MK3; Thermo Labsystems Inc., Beverly, MA, USA) at 540 nm to determine the quantities of viable cells. All experiments were performed in triplicate. Data were normalized to their respective controls and presented as a bar graph.

Detection of apoptosis by flow cytometry. Cell apoptosis was detected using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences). At 72 h post-transfection, the cells were collected, centrifuged three times at 1,000 \times g for 5 min, and resuspended in 500 μ l 1X binding buffer. The cell number was adjusted to 1 \times 10⁶ cells/ml. The cells (100 μ l) were placed in a tube, annexin V-FITC (5 μ l) and PI (5 μ l) were added and then the cell suspension was incubated for 15 min in darkness at room temperature. Staining of cells was immediately

quantified using a flow cytometer (FACSCanto™ II; BD Biosciences). For each determination, a minimum of 50,000 cells were analyzed. Each experiment was performed three times.

Western blot analysis. The cells were lysed in radioimmunoprecipitation assay buffer supplemented with a protease inhibitor (phenylmethylsulfonyl fluoride) and phosphatase inhibitors on ice for 15 min. The protein concentrations were determined using the bicinchoninic acid assay (Beyotime Institute of Biotechnology). A total of 30 μ g total proteins were separated using 10% SDS-PAGE (Beyotime Institute of Biotechnology) and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with skimmed milk (Beyotime Institute of Biotechnology) in PBS at room temperature for 1 h, and then incubated with specific antibodies (1:200 or 1:1,000 dilution) at 4°C overnight. On the following day, the membranes were washed three times with PBS, followed by incubation with HRP-conjugated secondary antibodies (1:5,000 dilution) at 37°C for 1 h. Following washing with

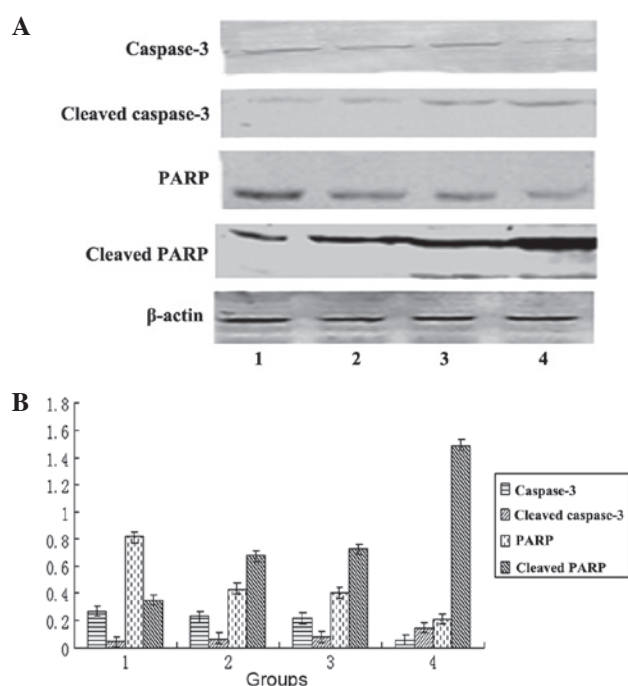


Figure 4. Co-transfection of β -catenin and STAT3 siRNAs synergistically promotes the cleavage of caspase-3 and PARP. HepG₂ cells were transfected with β -catenin siRNA and STAT3 siRNA alone or in combination using Lipofectamine™ 2000 for 72 h. (A) Immunoblots were performed to determine the protein levels of caspase-3, cleaved caspase-3, PARP and cleaved PARP, with β -actin as the loading control. (B) Quantification of A. Data were normalized to an internal control (β -actin) and expressed as the mean \pm standard deviation. The X-axis indicates the following: 1, Control group; 2, β -catenin transfection group; 3, STAT3 transfection group; 4, β -catenin and STAT3 co-transfection group. The Y-axis is the grayscale ratio of the protein of interest to the β -actin protein. * $P < 0.05$, compared with the control group; ** $P < 0.05$, compared with the control group and the groups transfected with β -catenin or STAT3 alone. STAT3, signal transducer and activator of transcription 3; siRNA, small interfering RNA; PARP, poly(ADP-ribose) polymerase.

PBS, the bands were developed in a solution of 3,3'-diaminobenzidine (Santa Cruz Biotechnology, Inc.). Once the bands were visible on the membranes, the color development was stopped and the membranes were rinsed in distilled water. Images were captured using a gel imaging system (Champgel 5500; Beijing Sage Creation Science and Technology Ltd., Beijing, China) and analyzed using Quantity One 4.5.2 software (Bio-Rad, Hercules, CA, USA). The quantitative results of gray-scale analysis were subjected to statistical analysis.

Statistical analysis. Values are expressed as the mean \pm standard deviation, and were compared using the Student's t-test and analysis of variance. Statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Efficient downregulation of β -catenin and STAT3 proteins by siRNAs in HepG₂ cells. Following siRNA transfection in HepG₂ cells for 72 h, the β -catenin and STAT3 protein levels were determined by western blot analysis. β -catenin was efficiently decreased in the β -catenin transfection group and

in the β -catenin and STAT3 co-transfection group after 72 h ($P < 0.05$). Similarly, STAT3 siRNA downregulated STAT3 protein expression in the STAT3 transfection group and in the β -catenin and STAT3 co-transfection group ($P < 0.05$; Fig. 1). Accordingly, HepG₂ cells transfected with these siRNAs for 72 h were used for subsequent analysis.

Simultaneous silencing of β -catenin and STAT3 by siRNAs enhances the loss of HCC cell viability. The cell viability of HepG₂ cells was determined following β -catenin and STAT3 silencing with siRNAs using an MTT assay. The cell viability in the control group exhibited no significant difference compared with that in the negative siRNA control group; however, the transfection group exhibited a significant decrease in the number of viable cells ($P < 0.05$). No significant difference was identified in the number of viable cells between the β -catenin and STAT3 transfection groups. By contrast, the β -catenin and STAT3 co-transfection group revealed a more marked decrease in cell viability than the β -catenin or STAT3 transfection groups ($P < 0.05$; Fig. 2). The cell growth inhibition ratios were 14.2% at 24 h, 19.6% at 48 h and 27.6% at 72 h (β -catenin transfection group); 11.4% at 24 h, 17.6% at 48 h and 24.9% at 72 h (STAT3 transfection group); and 19.6% at 24 h, 27.1% at 48 h and 39.9% at 72 h (β -catenin and STAT3 co-transfection group). These results revealed that the growth inhibition rate in the β -catenin and STAT3 co-transfection group was greater than that in the groups transfected with β -catenin or STAT3 alone, particularly at 72 h. These results suggested that simultaneous inhibition of β -catenin and STAT3 resulted in an enhanced reduction of the cell viability of liver cancer cells.

Co-transfection of siRNAs targeting β -catenin and STAT3 increases cell apoptosis in HCC cells. Cell apoptosis was assessed following siRNA transfection for 72 h using flow cytometry. Compared with that the control group, the early apoptotic rates of the three transfection groups were significantly increased ($P < 0.05$). However, the early apoptotic rate was not significantly different between the β -catenin siRNA and STAT3 siRNA transfection groups. In addition, the early and late apoptotic rates of the β -catenin and STAT3 co-transfection group revealed a more marked increase than those of the β -catenin or STAT3 transfection group alone ($P < 0.05$; Fig. 3).

Simultaneous downregulation of β -catenin and STAT3 by siRNAs synergistically promotes the cleavage of caspase-3 and PARP. Finally, the protein expression levels of the cell apoptosis markers caspase-3, cleaved caspase-3, PARP and cleaved PARP were determined using western blot analysis following siRNA transfection for 72 h. The caspase-3 and PARP protein levels decreased in the three transfection groups, while no significant difference was observed between the β -catenin and STAT3 transfection groups alone. However, the caspase-3 and PARP protein levels were more markedly decreased in the β -catenin and STAT3 co-transfection group ($P < 0.05$). By contrast, the cleaved caspase-3 and cleaved PARP protein levels increased in the three transfection groups; however, they exhibited no significant difference between the β -catenin and STAT3 transfection groups alone. Increased

cleavage of caspase-3 and PARP protein was clearly observed in the β -catenin siRNA and STAT3 siRNA co-transfection group ($P < 0.05$; Fig. 4).

Discussion

It has been well documented that the β -catenin and STAT3 signaling pathways are important in the evasion of apoptosis of cancer cells (15-18). The final critical stage of apoptosis is achieved through caspase activation, in which the role of caspase-3 is particularly important. PARP, a cell death substrate, protects the structural integrity of chromosomes and is involved in DNA repair (19). PARP was the first identified substrate of caspase-3 during apoptosis and is the most characteristic proteolytic substrate (20). Following DNA damage, PARP is activated, and rapidly recognizes and binds to damaged DNA sites to promote DNA repair (21).

In the present study, β -catenin and STAT3 protein expression levels were markedly inhibited by siRNA transfection for 72 h, demonstrating that the transfection was effective. Silencing β -catenin and STAT3 gene expression inhibited HCC cell growth and promoted apoptosis. In addition, silencing β -catenin and STAT3 gene expression decreased the caspase-3 and PARP protein levels and increased the cleavage of caspase-3 and PARP proteins. The decrease in the expression of PARP would impair DNA damage repair, thereby promoting cell apoptosis. Similar to the present findings, in human squamous cell lung carcinomas, overexpression of the protein inhibitor of activated STAT3 promoted mitochondrial depolarization, leading to cytochrome C release, caspase-9 and caspase-3 activation, as well as PARP cleavage (22). In glioma cells (23), ovarian cancer cells (24), a urethane-induced lung tumor model (25), human renal cell carcinoma and melanoma cell lines (26), inhibition of STAT3 activity has been revealed to elevate the levels of cleaved caspase-3 and PARP, which is concordant with the results of the present study. In addition, β -catenin signaling is involved in the regulation of cell growth and apoptosis in breast cancer (27). Accordingly, inhibition of β -catenin signaling induces apoptosis through activation of caspase-3, induction of B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), inhibition of Bcl-2 and cleavage of PARP in gastric cancer cells (28). In addition, significant dephosphorylation of glycogen synthase kinase-3 β at serine 9 reduced the activation of caspase-3, as well as Bax and PARP cleavage in human neural progenitor cells (29).

In the present study, the alterations in caspase-3, cleaved caspase-3, PARP and cleaved PARP protein levels in the β -catenin and STAT3 co-transfection group were more marked than those in the β -catenin or STAT3 siRNA alone transfection group. In addition, the ratios of liver cancer cell growth inhibition and apoptosis in the β -catenin and STAT3 co-transfection group were significantly greater than those in the groups transfected with β -catenin or STAT3 siRNA alone. Similar to the results of the present study, simultaneous silencing of vascular endothelial growth factor, telomerase reverse transcriptase and Bcl-extra large expression improved the inhibition of cell growth and promotion of apoptosis in laryngeal squamous carcinoma (30).

The tumorigenesis of liver cancer is the result of the collaboration of multiple pathways. Inhibiting the functions of the

key genes of several pathways together is expected to achieve improved results for the purpose of cancer therapy (30). In agreement with this hypothesis, it was demonstrated that silencing β -catenin and STAT3 genes together led to enhanced cell apoptosis and loss of cell viability of HCC cells as compared with silencing of β -catenin and STAT3 genes individually. The present study provided proof-of-concept that targeting several key components of different pathways together may be a potential strategy for future development of anti-cancer drugs.

In conclusion, it was identified that simultaneous silencing of β -catenin and STAT3 by siRNAs resulted in enhanced loss of cell viability and induction of apoptosis of liver cancer cells. The present study provides a basis for further preclinical and clinical development of anti-cancer therapeutic strategies targeting several cancer growth-promoting signaling pathways simultaneously.

Acknowledgements

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